Small Peptides as Potent Mimetics of the Protein Hormone Erythropoietin

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Random phage display peptide libraries and affinity selective methods were used to isolate small peptides that bind to and activate the receptor for the cytokine erythropoietin (EPO). In a panel of in vitro biological assays, the peptides act as full agonists and they can also stimulate erythropoiesis in mice. These agonists are represented by a 14–amino acid disulfide-bonded, cyclic peptide with the minimum consensus sequence YXCXXGPXTWXCXP, where X represents positions allowing occupation by several amino acids. The amino acid sequences of these peptides are not found in the primary sequence of EPO. The signaling pathways activated by these peptides appear to be identical to those induced by the natural ligand. This discovery may form the basis for the design of small molecule mimetics of EPO.

The binding of cytokines to their receptors occurs over large surfaces on both the ligand and receptor, as exemplified by the crystal structure of growth hormone (GH) bound to its receptor. The structure of the GH complex shows that more than 30 side chains on the hormone surface and on each of the two receptor subunits are buried on binding (*1*). Of these many residues, however, only three, clustered in a very small area on each receptor, contribute 75 percent of the binding energy (*2*). If proteins generally bind one another through small epitopes, the potential for finding small molecule ligands is good.

New methods for ligand discovery are based on combinatorial procedures for assembling large numbers of compounds to produce diverse molecular shapes testable for binding molecular targets (*3*, *4*). Because of the numbers of compounds produced, it is necessary to employ methods for testing the compounds in pools, rather than individually. Vast libraries of peptides can be created through cloning complex mixtures of combinatorially synthesized oligonucleotides into specialized expression "display" vectors. The filamentous phage display system, whereby the expressed peptides are displayed as fusions to phage coat proteins, has been effective in the discovery of ligands (*5*–*8*). Affinity purification of the population of phage particles on the target protein is used to recover peptides with binding activity. Sequencing the appropriate segment of the DNA of each captured phage provides the primary sequence of peptides that bind the target.

We used the filamentous phage method to search for peptides that would bind to the extracellular domain of the human EPO receptor (EPOR), and to derive from such ligands compounds capable of stimulating erythropoiesis. The primary regulator of erythropoiesis, which is a complex process of cellular differentiation and proliferation resulting in the production of red blood cells, is the glycoprotein hormone EPO (*9*, *10*). Human EPO is a 34-kD monomeric molecule composed of 165 amino acid residues (18.5 kD) and an almost equivalent mass of carbohydrate. The structure of EPO is predicted to be a bundle of four α helices, sharing common secondary structure motifs and tertiary fold with other members of the family of hematopoietic growth factors of known three-dimensional structure (*11*, *12*).

The biological effects of EPO are exerted through binding to the EPOR, which is expressed on the surface of target cells (*13*, *14*). The EPOR belongs to the hematopoietic cytokine receptor family, which includes receptors for most of the interleukins and various other hematopoietic and nonhematopoietic growth factors (*12*, *15*, *16*). These receptors are type I cell surface proteins composed of an NH_2-terminal ligand binding domain, a COOH-terminal cytoplasmic region, and a single membrane-spanning domain. Conserved features of the extracellular domain include two pairs of cysteine residues and a "WSXWS" motif (*17*) with characteristic spacing. The receptor is believed to become activated after homodimerization (*18*–*20*), possibly by the mechanism established for GH receptor (*1*, *21*), where the monomeric hormone molecule binds to and causes the dimerization of two identical receptor chains. This proposed mechanism is supported, in part, by the finding that a constitutively active EPOR containing a single point mutation (R129C) in the extracellular domain exists as a homodimer (*22*). Although the receptor shows no intrinsic kinase activity, protein tyrosine phosphorylation plays a pivotal role in EPOR signal transduction (*23*, *24*), through the activation of receptor-associated tyrosine kinases (*25*, *26*).

Recombinant human EPO has a role in the treatment of anemia associated with certain pathological conditions (*27*, *28*). As a glycoprotein, EPO must be administered either by intravenous or subcutaneous injection. In seeking smaller molecules to serve as lead compounds in the development of a more conveniently delivered agent, and to acquire structural probes of the receptor, we characterized the recovered peptide ligands for EPOR binding and specificity, and for EPO-mimetic properties in receptor activation assays. We showed that these peptides have the ability to specifically activate an erythropoietic response in cell-based assays and in animals. The structure of a complex of one of these peptides with the EPOR has been described by Livnah *et al.* (*29*) and elucidates the mechanism by which receptor activation is achieved.

Identification and optimization of ligands to EPOR. The isolation of peptides that bind and activate the EPOR was facilitated by the availability of a soluble form of the EPOR extracellular domain (ECD) that binds EPO with an affinity similar to that reported for the full-length receptor (*13*, *14*). We expressed the EPOR ECD in Chinese hamster ovary cells as a fusion protein to aid in purification and immobilization by a method that has been used for other type I receptors (*30*–*32*). The COOH-terminal signal sequence of the human placental alkaline phosphatase (HPAP) was fused to the carboxyl terminus of the EPOR ECD. This signal sequence directed the expression of the truncated receptor to the cell surface where it is anchored by a phosphatidylinositide glycan linkage. Soluble EPOR (sEPOR) was obtained by treating the cells with phospholipase C and was immobilized in polystyrene wells by a monoclonal antibody (mAb) to the HPAP domain

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(mAb179). The dissociation constant (K_d) of recombinant human EPO (rhEPO) for this immobilized sEPOR was about 200 pM, a value similar to that found for the native receptor expressed on cells of the erythroid lineage (*13*) (Fig. 1A).

Initial attempts to isolate EPOR-specific clones relied on acid elution to recover phage and were unsuccessful. We therefore modified the sEPOR by the insertion of a protease (thrombin) cleavage site between an the ECD and HPAP domains. This modification, which had minimal effect with respect to ligand binding, allowed us to use thrombin cleavage instead of acid elution to liberate only those phage particles bound to the sEPOR. Using the thrombin release procedure (*33*), we isolated a receptor-specific clone (designated C2/11) from a library of cyclic 8-residue peptides displayed on pVIII (*34*). An enzyme-linked immunosorbent assay (ELISA) was used to demonstrate EPOR-binding activity. This clone did not bind to mAb179, bovine serum albumin (BSA) or to the extracellular domains of the receptors for tumor necrosis factor (TNFR-p55), nerve growth factor (NGFR), interleukin-2 (IL-2Rα, -β, and -γ subunits) and E-selectin (*35*). Furthermore, EPO was found to compete with clone C2/11 for binding to the immobilized sEPOR (*35*).

The DNA insert encoding the peptide displayed by clone C2/11 was sequenced, and the peptide sequence was deduced to be CRIGPITWVC (*17*). This peptide, designated AF11154, was synthesized to include two pairs of glycine residues flanking the library peptide sequence (as contained in all peptides in the library) and oxidized to the intramolecular disulfide-bonded (cyclic) form. The structure of the purified synthetic peptide was confirmed by mass spectrometry. This peptide was capable of competing with ^{125}I-labeled EPO binding to immobilized sEPOR with an apparent affinity of approximately 10 μM (Table 1 and Fig. 1B). This confirmed the ability of the peptide to bind the receptor when removed from the context of fusion with pVIII. Furthermore, the peptide did not compete for binding of either human IL-1β to the human IL-1 type II receptor, or human IL-2 to the human IL-2 receptor αβ hetero-dimer (*35*).

To obtain peptides with higher affinity, we performed phagemid display mutagenesis (*30, 32*). We used the lower valency pIII phagemid display system in which 0 to 5 copies of fusion protein are expressed per particle, in contrast to 100 to 200 copies of peptide displayed in the pVIII system. Mutagenesis libraries were screened with the use of an EPO elution step to enrich for higher affinity clones (*32, 36, 37*). An initial round of screening was carried out without EPO competition in order to establish a pool of receptor-specific clones. In subsequent rounds, EPO was added after phagemid particles were allowed to bind to the sEPOR. After the lower affinity clones were removed by washing, the remaining phagemid particles were recovered by acid elution. The stringency of EPO elution was increased progressively in subsequent rounds of screening as the ligand concentration, temperature, and duration of incubation were increased (*37*).

Two mutagenesis libraries were constructed. For the first library, ON1962, oligonucleotides were synthesized so that each amino acid of the RIGPITWV sequence was independently substituted by any other amino acid; in theory, a substitution at each position would occur at a frequency of 50 percent. Since the parental sequence, when expressed as a pIII fusion, does not bind the EPOR (*35*), any peptides isolated in the mutagenesis screening were likely to be of higher affinity for this receptor. No EPOR-specific clones were obtained from screening this library, and therefore we constructed a second mutagenesis library containing peptides of increased length in order to expand the number of possible receptor contacts, potentially increasing binding affinity. Library ON1963 was constructed as above, except that three additional random amino acids were added on each side of the cysteine-bounded core sequence. In contrast to our result with ON1962, this library yielded a large number of sEPOR-specific phagemid clones. The DNA inserts were sequenced and translated, identifying a family of related peptides (nearly

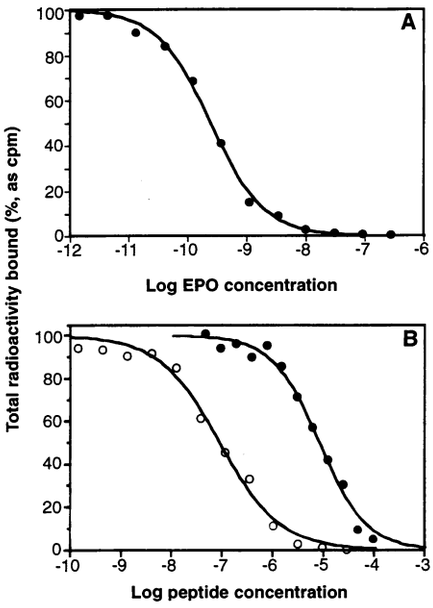

Fig. 1. ^{125}I-EPO competition binding assays. (**A**) Determination of binding affinity of immobilized sEPOR for recombinant human EPO (rhEPO). The binding assay was performed as described (*30*), with modifications. All steps were performed in RPMI media containing 1 percent bovine serum albumin (BSA), except for washes, where phosphate-buffered saline (PBS) was used. sEPOR was immobilized in microtiter strip wells and incubated with radioiodinated rhEPO (Amersham International—300 to 900 Ci/mmol). rhEPO (*59*) was used as competitor and wells were washed after a 2-hour incubation at 4°C, separated and bound counts determined. Nonspecific binding was measured in the presence of 1 μM rhEPO. Data was analyzed using the ALLFIT program (*60*). (**B**) IC_{50} plots of two EPOR peptide ligands. Binding assays were performed essentially as described above. Peptides were dissolved in 100 percent dimethylsulfoxide (DMSO, Sigma) at 50 mM and diluted in binding buffer to the starting concentrations. The maximum DMSO concentration in any assay was 0.4 percent. Peptide AF11154 (closed circles) and EMP1 (open circles).

Table 1. Peptides synthesized for characterization in EPOR binding assays and EPO bioassays. Peptides were made in the cyclic (disulfide-bonded) form and amidated at the COOH-terminal. Correct structure of purified peptides (>95 percent pure, as judged by high-performance liquid chromatography) was confirmed by mass spectrometry. The conserved C and Y residues (*17*) and GPXTW motifs are shown in bold. Flanking G residues were derived from vector sequences. Concentrations required for 50 percent inhibition of tracer ^{125}I-labeled EPO binding (IC_{50} values) were determined in a competition binding assay (Fig. 1). Under the assay conditions, the IC_{50} was approximately equal to the affinity (K_d).

Peptide	Sequence	IC_{50} (μM)
AF11154	GG**C**R**IGPITWVC**GG	10.0
EMP1	GGT**Y**S**C**HF**GPLTWVC**KPQGG	0.2
EMP2	GGD**Y**H**C**RM**GPLTWVC**KPLGG	0.2
EMP3	GGV**Y**A**C**RM**GPITWVC**SPLGG	0.3
EMP4	VGN**Y**M**C**HF**GPITWVC**RPGGG*	0.5
EMP5	GGL**Y**L**C**RF**GPVTWDC**GYKGG	1.0

EYKCYMGPITWVCKPE NYTCRFGPLTWECTPQ
DYTCRMGPMTWICTAT SWDCRIGPITWVCKWS
NYLCRFGPGTWDCTGF NYMCHFGPITWVCRPG
NYVCRMGPITWICTPA LYLCRMGPQTWMCQPG
SYLCRMGPTTWLCTAQ DYVCRMGPMTWVCAPY
DYTCRMGPMTWICTAT WYSCLMGPMTWVCKAH
EYSCRMGPMTWVCSPT YYYCWMGPMTWVCSPA
EYLCRMGPITWVCERY CVMCRIGPITWVCDIP
LYLCRFGPVTWDCGYK NLQCCIGPITWVCRHA
TYSCHFGPLTWVCKPQ EYFCRMGPITWVCQRS
IYKCLMGPLTWVCTPD LYICRMGPLTWECKRT
DYHCRMGPLTWVCKPL QYACRMGPITWVCKYM
LYECRMGPMTWVCRPG VYLCTFGPITWLCRGA
LYLCRMGPVTWECQPR NYACRMGPITWVCSPL
DYNCRFGPLTWVCKPS LYYCRFGPITFECHPT
QLLCGIGPITWVCRWV LYACHMGPMTWVCQPL
KYSCFMGPTTWVCSPV LYTCRMGPITWVCLPA

Fig. 2. Peptides expressed on clones recovered from mutagenesis library ON 1963. All clones retained at least 60 percent of total binding in wells incubated with EPO, as described (*38*). Residues showing a high degree of conservation are shown in red. A proline residue, conserved to a lesser degree, is shown in green.

100) (Fig. 2). Because of the quantity of EPOR-binding clones isolated from this library, we devised a strategy to identify those of higher affinity. We developed an elution ELISA format in which EPOR-bound phagemids were allowed to dissociate in the presence of EPO (*38*). The reduction in signal when compared to a noneluted sample was used as a possible indication of ligand affinity. Five peptides chosen on the basis of their signals in this assay were synthesized in the cyclic (disulfide-bonded monomer) form (Table 1). The affinities of these peptides in the immobilized EPOR binding assay were established (Table 1), and the peptides showed a 10 to 50 times higher affinity than that shown by the parental peptide ligand AF11154 (Fig. 1B). The importance of the disulfide bond was demonstrated by testing linear analogues of the peptides in which either or both of the cysteine residues were mutated to serine. These were at least 1000 times lower in binding affinity (*35*).

The five peptides chosen for synthesis are representative of two highly conserved features within the family of sequences. The first, arising within the mutated core sequence between the fixed cysteines, is the absolute conservation of GPXTW motif, suggestive of a β-turn motif. The almost perfect conservation of a tyrosine outside the disulfide loop was also reflected in the peptides synthesized for testing. It is probable that the selection of this tyrosine residue accounted, in part, for the substantial increase in affinity for the sEPOR. Furthermore, the frequency of proline in the COOH-terminal flanking sequence indicates its possible importance as a binding determinant. These hypotheses are supported by the crystal structure of a co-complex of a representative EPOR agonist peptide with the extracellular domain of the human EPOR (*29*). This structure confirms that the GPLT sequence resides in a β turn that also makes contacts with the receptor. The tryptophan residue of the GPXTW motif contributes to stabilization of the peptide structure, whereas the tyrosine constitutes an additional, important receptor contact point.

EPO-mimetic activity of peptides in biological assays in vitro and in vivo. To evaluate the biological potential of the peptides on EPO-responsive eukaryotic cell lines, we constructed an EPO-responsive line by transfecting FDCP-1 cells with the full-length human EPOR, as described for the murine EPOR (*39*). EPO stimulates this line to proliferate, with half maximal response (EC_{50}) occurring at 10 to 20 pM (Fig. 3A). The parental line did not respond to EPO, but retained the growth requirement of a supplement of WEHI-3 conditioned media (*40, 41*). The low affinity peptide ligand, AF11154, was inactive in this assay at concentrations up to 200 μM (*35*). However, the higher affinity peptides were found to be full EPOR agonists, supporting cell proliferation and producing the same maximal proliferative response as EPO (Fig. 3B). For this reason the nomenclature "EPO mimetic peptide" (EMP) was adopted. The peptides did not activate the FDCP-1 parental cell line, thereby demonstrating that the proliferative effect was dependent on the presence of the EPOR (*35*). Two peptides, EMP4 and EMP5, produced amounts of cell proliferation in excess of the maximum reached by EPO. The reason for this is unclear although of the five peptides tested for agonist activity in this assay, these two were only partially soluble at the higher end of the concentration range. EMP1, EMP2, and EMP3 were freely soluble across the range of concentrations tested and each reached the same plateau as EPO in the proliferation assay. The potency of the peptides was substantially lower than that of EPO, their EC_{50}'s being from 200 nM (EMP2 and EMP3) to 400 nm (EMP1), compared to 20 pM for the natural ligand.

To confirm that the activity was due to the peptide and not the result of any contamination by EPO, we performed a proliferation assay with EMP4 and FDCP-1/hEPOR cells in which a rabbit polyclonal EPO neutralizing serum was superimposed at a 1 percent concentration on top of both standard EPO and peptide curves. The activity of EPO was almost totally inhibited by the antiserum. The proliferative response to EMP4, however, was not diminished by the antiserum (*35*). The EPO-mimetic bioactivity of EMP4 and EMP5 on the murine EPOR (mEPOR) was also assayed with FDCP-1 cells transfected with the full-length mEPOR (FDCP-1/mEPOR). Both were active on these cells (*35*).

One of the more active peptides, EMP1,

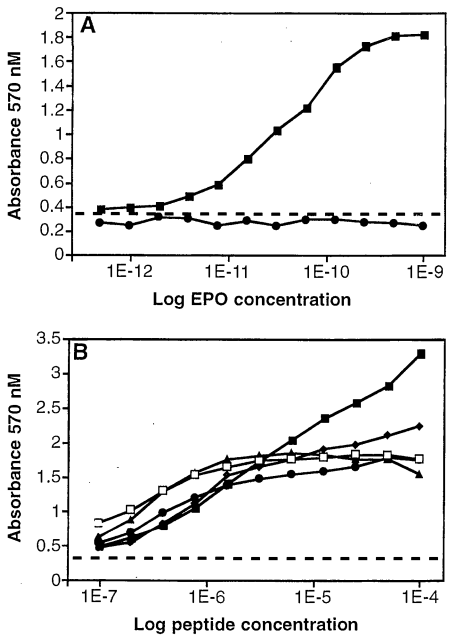

Fig. 3. FDCP-1/hEPOR cell proliferation assays. (**A**) EPO curve. FDCP-1/hEPOR cells were grown to mid-log phase (10^6 cells per milliliter) in RPMI containing 10 percent fetal calf serum (FCS) supplemented with 1 nM rhEPO (*59*). The cells were then washed free of rhEPO with PBS and cultured (overnight) in media that did not contain EPO. Untransfected cells, grown in 10 percent WEHI-3 conditioned media (*40*), were treated similarly. rhEPO was administered to the cells (10^5 cells per well) at the indicated concentrations. Cell proliferation was determined after 48 hours with the use of a colormetric assay (*61*). FDCP-1 cells (circles) and FDCP-1/hEPOR (squares) in the presence of EPO. Dashed line indicates proliferation in absence of EPO. (**B**) Proliferative response of FDCP-1/hEPOR to peptides. Peptides were dissolved in 100 percent DMSO at 50 mM and diluted to starting concentrations in serum-free RPMI. EMP1 (closed circles), EMP2 (triangles), EMP3 (open squares), EMP4 (diamonds), and EMP5 (closed squares).

Table 2. Effect of EMP1 on erythroid colony formation from human bone marrow cells. Human nucleated bone marrow cells obtained from normal donors (*57*) were plated in semisolid methyl cellulose cultures (*58*) in the presence of EMP1, rhEPO, or a combination of recombinant human growth factors IL-3, GM-CSF, and SCF (3/GM/SCF) (*58*) at the concentrations shown. Erythroid and granulocyte-macrophage (G-M) colonies formed after 12 days of culture were scored by color (erythroid colonies appear red) and morphology.

Growth factor	Concentration (nM)	Number of colonies per 2×10^5 nucleated cells			
		Erythroid		G-M	
		Plate 1	Plate 2	Plate 1	Plate 2
None*		0	0	0	0
EPO	(0.24)	113	106	12	9
EMP1	(1000)	29	23	1	2
EMP1	(10,000)	80	75	2	0
3/GM/SCF	(0.7/0.7/2.7)	0	0	176	212

*No factors.

was chosen for further study. First, the ability of EMP1 to stimulate proliferation was analyzed in cell lines with endogenous EPOR that are EPO-responsive. The peptide stimulated both TF-1 (*42*) and B6Sut (*43*) cells in a dose-dependent manner, but did not show proliferative activity on other myeloid cell lines that are unresponsive to EPO, including MO7e (*44*) and AML-193 (*45*), indicating the effect to be erythroid-lineage specific (*41*). Furthermore, EMP1 did not have proliferative potential on the nonmyeloid cell lines T47D (*46*) and MC3T3 (*47*), which are stimulated by progestin and serum, respectively (*41*). A further indication of the peptide specificity for erythropoietin receptor-bearing cell lines was its lack of activity on a human T cell clone (*41*) that is responsive to interleukin-2 (*48*).

To assess the activity of EMP1 in supporting the expansion and differentiation of cells of the erythroid lineage, we performed colony assays with human bone marrow. Cells were plated in methylcellulose medium containing either peptide (1 μM and 10 μM), rhEPO (0.24 nM), or a combination of the recombinant human growth factors interleukin-3 (IL-3, 0.7 nM), granulocyte-macrophage colony-stimulating factor (GM-CSF, 0.7 nM), and stem cell factor (SCF, 2.7 nM). A control culture received no additional factors or peptide. Plates were incubated for 12 days, after which erythroid colonies (CFU-E) and granulocyte-macrophage colonies (CFU-GM) were counted (Table 2). No colonies grew in the absence of additional factors or peptide. In cultures receiving peptide alone, the production of erythroid colonies was supported in a dose-dependent manner. The effect of the peptide was shown to be erythroid-specific by the effective absence of CFU-GM in these cultures compared to the control containing factors known to support their growth (IL-3, GM-CSF, and SCF). This is in agreement with the cell culture results in which EMP1 did not stimulate MO7e (*44*) or AML193 (*45*), two cell lines that respond to GM-CSF, but not to EPO. EMP1 was also active in promoting erythroid colony formation from human peripheral blood and murine bone marrow (*41*).

An examination of the specific and temporal intracellular events induced on stimulation with either EPO or EMP1 was undertaken to determine if similar signaling pathways were utilized by both the natural and mimetic EPOR ligands. The binding of EPO to its receptor causes rapid phosphorylation of cellular substrates on tyrosine residues, most likely due to the activation of JAK2 (*25, 26*). A number of proteins phosphorylated on tyrosine residues after EPO stimulation have been identified (*25, 49–51*). The pattern of protein tyrosine phosphorylation following a 10-minute stimulation of FDCP-1/mEPOR cells by EPO or EMP1 was examined by Western blot analysis utilizing an anti-phosphotyrosine monoclonal antibody. EPO induced the phosphorylation of proteins with apparent molecular weights of 140, 95, 70, and 55 kD (Fig. 4). These proteins most likely correspond to JAK2 (*25*), vav (*49*), EPOR (*51*), and shc (*50*), respectively. When compared to EPO, EMP1 produced an identical phosphorylation pattern.

Another indication that EMP1 and EPO use same signaling pathway was the finding that similar cell-cycle kinetics were induced by both ligands. Factor-starved FDCP-1/mEPOR cells were stimulated with either EPO or EMP1 and analyzed by propidium iodide staining and flow cytometry at the indicated time intervals (Fig. 5). After 18 hours in the absence of either factor, the majority of the cells were in G_0/G_1. Eight hours after the cells were stimulated, approximately 35 percent were in S phase in both EPO and peptide-treated cultures. Continued incubation (10 and 12 hours) and analysis of cells treated with either agent resulted in comparably increased progression into S phase.

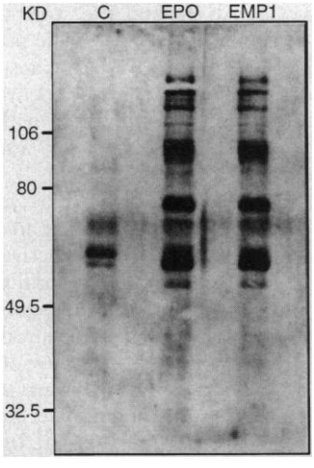

Fig. 4. Phosphotyrosine analysis. FDCP-1/mEPOR cells (FDCP-1 transfected with full-length murine EPOR) were grown to stationary phase (2×10^6 cells per milliliter) in RPMI containing 10 percent FCS, 2 nM rhEPO, harvested, washed and resuspended in fresh medium without growth factor (rhEPO) for 24 hours. Cells were counted and resuspended at a concentration of 0.5×10^6 cells per milliliter. One milliliter of cells was then stimulated for 10 minutes either with rhEPO (10 U/ml) or EMP1 (10 μM). Control cells received no stimulation. Patterns of intracellular protein tyrosine phosphorylation were determined by immunoblotting, probed with a monoclonal antibody to phosphotyrosine (4G10, Upstate Biotech.) as described (*51*).

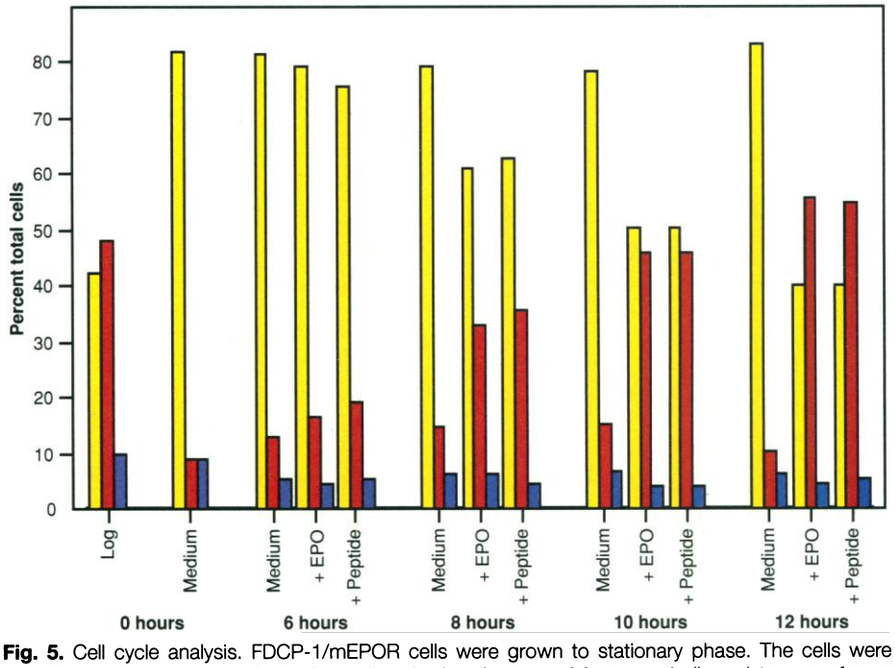

Fig. 5. Cell cycle analysis. FDCP-1/mEPOR cells were grown to stationary phase. The cells were collected and resuspended in fresh medium in the absence of factor and allowed to grow for an additional 18 hours. Cells were then stimulated with either EPO at 10 U/ml or 10 EMP1 at 10 μM, or not treated. Three million cells were collected at 0, 6, 8, 10, and 12 hours. Cells were washed twice in cold PBS, centrifuged, and then fixed in 100 μl of cold PBS to which 900 μl of cold 70 percent ethanol was then added. The cells were stained with propidium iodide (50 μg/ml), and fluorescence was measured on a flow cytometer (FACScan). The percentage of cells in each phase was measured with the SOBR model of CellFIT software (Beckton Dickinson). Percent G0/G1 phase, yellow columns; percent S phase, red columns; percent G2/M phase, blue columns.

EMP1 was tested in vivo for EPO mimetic activity in two murine models of erythropoiesis. The first was an assay in which normal, untreated mice containing endogenous basal levels of EPO were used (*52*). Reticulocyte counts (erythrocyte precursors) in peripheral blood were determined by flow cytometry after mice were treated with various amounts of either EPO or peptide (Fig. 6A). At the highest doses of EPO tested (40 units), the increase in reticulocytes was approximately 15 percent. A smaller, but significant increase of approximately 6 percent resulted from the administration of peptide (2 mg), demonstrating that EMP1 can induce the production of reticulocytes over the background of endogenous EPO in this animal model. A second, more sensitive assay, the exhypoxic-polycythemic mouse bioassay, was used to assess further the in vivo activity of the peptide (*53*). This assay relies on the induction of polycythemia by conditioning mice in a hypobaric chamber to suppress endogenous EPO production. Increases in ^{59}Fe incorporation into blood can be attributed to exogenously administered erythropoietic agents. A dose response was produced over the range of EPO administered (0.025 to 0.100 U) (Fig. 6B). At the highest dose of EPO, approximately 16 percent of total counts were incorporated (vehicle and DMSO backgrounds 1 percent and 2 percent, respectively). EMP1 also showed a dose-dependent response in this assay, with detectable activity over the entire range tested (1.0 to 54.0 μg per mouse). At a dose of 54 μg of peptide per mouse, the amount of ^{59}Fe incorporated was equivalent to that observed for 0.10 U of EPO per mouse.

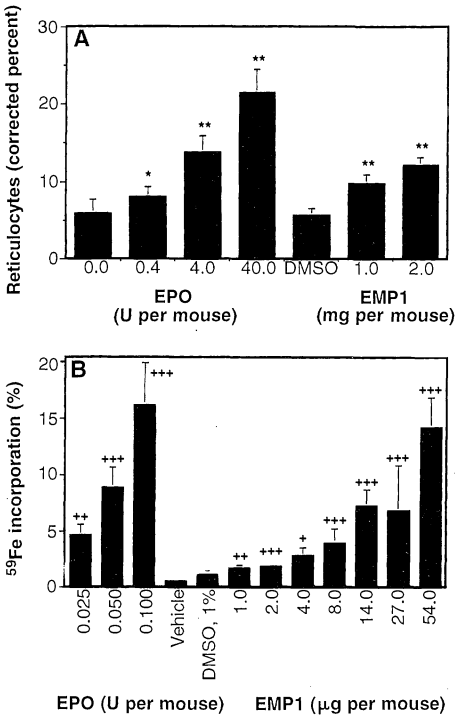

Fig. 6. Biological activity of EMP1 in vivo (*62*). (**A**) Murine reticulocyte assay. The protocol, including dosing regimen, is essentially as described previously (*52*). There were 10 mice in each group, unless noted otherwise. Peptide samples were solubilized in DMSO and subsequently diluted with assay vehicle. Hematocrits and reticulocyte counts were determined and deviations in hematocrit corrected as described (*52*). For dose groups 1.0 and 2.0 mg of peptide, $N = 8$. Mean values are shown, $^{*}P < 0.01$; $^{**}P < 0.0001$. (**B**) Exhypoxic Polycythemic Mouse Bioassay. The method was adapted from (*53*). Peptide or EPO were diluted in PBS containing 0.1 percent BSA. Mice ($N = 10$ per group except 4 μg $N = 9$) were injected with a single dose of sample as indicated. The percentage of ^{59}Fe incorporated was determined (*63*). Mean values are shown, $^{+}P < 0.03$, $^{++}P < 0.001$, $^{+++}P < 0.0001$).

Implications of these findings. We have isolated and characterized a family of cyclic peptides with erythropoietic activity. We initially identified an eight–amino acid peptide sequence by screening libraries of random peptides displayed on filamentous phage. This sequence served as the starting point for the development of a family of larger, closely related peptides with enhanced binding affinity and EPOR agonist activity. None of the EPOR peptide ligands that we identified had homology with the primary sequence of EPO. The EPOR-dependent agonist activity was demonstrated in various cell lines containing either the recombinant or native EPO receptors of both human and murine origin. A representative peptide (EMP1) was found to induce a pattern of tyrosine phosphorylation indistinguishable from that produced by stimulation with human EPO. That these properties were mediated through the EPOR and were not a result of general mitogenic effects was shown by the inability of the peptide to promote growth of cells responsive to factors other than EPO. Remarkably, these agonist properties extend to in vivo models of erythropoiesis.

Phage display technology proved to be instrumental in both the initial discovery of the peptide ligand and the selection of higher affinity agonists. Essential to this process was a receptor engineered to permit the specific release of receptor-phage complexes by proteolytic release of immobilized receptor. Despite exhaustive screening of many libraries, we recovered receptor-specific ligands only from a library of disulfide-cyclized peptides. Furthermore, linear analogues of the active cyclic peptides made by mutagenizing either or both of the flanking cysteine residues to serine had a binding affinity that was at least 1000 times lower and exhibited undetectable agonist activity (*35*).

A surprising property of these peptides was the ability to activate a growth factor receptor. A growing body of evidence implicates the role of cytokine-mediated receptor dimerization in the initiation of signal transduction, as initially shown for growth hormone receptor activation (*1*). Here we have shown that peptides of approximately 2 kD can bind in the EPO binding site of the EPOR and mimic the action of the 34-kD growth factor. Furthermore, non–disulfide-mediated, covalently linked dimeric forms of EMP1 (made by means of two different strategies) have significantly increased potency in the EPO-dependent proliferation assay (*35*, *54*, *55*). Selection from the initial library and subsequent analog libraries was based solely on receptor binding and not receptor activation; however the process recovered peptides that not only bound but also triggered a receptor response indistinguishable from that produced by EPO. A possible explanation of this result is that immobilization of the receptors at high density on an array of immunoglobulin G molecules presents the ligand binding sites in close proximity, allowing the capture of ligands contacting both receptor molecules simultaneously.

When bound to a bacterially expressed form of the EPOR ECD (*56*), the peptides promote the formation of diffractable crystals of the complex. Livnah *et al.* (*29*) describe the structure of this complex at a resolution of 2.8 Å; and the structure reveals the mechanism by which the peptides can induce the erythropoietic response. Two peptide molecules, each contacting both receptor subunits, interact with one another in a nearly symmetric array to dimerize the receptors.

The EPO-mimetic peptide has yet another property; the ability to elicit an erythropoietic response in vivo. This activity did not depend on either a special formulation or chemical modification of the peptide. In addition, the dosing regimens for the in vivo bioassays were based on protocols previously established for EPO, and may not be optimal for administration of the peptide. Because of the commonly accepted generalization that L-peptides are rapidly degraded in serum, our finding of activity in the animals was unexpected. We have determined that in this case the generalization does not apply; the half-life of isolated EMP1 in 50 percent fresh mouse serum is approximately 8 hours (*54*). This stability may account, in part, for the ability of the peptide to exert its effect in vivo. Nevertheless, the peptide is less potent, compared with EPO, in the mouse than would be expected on the basis of its activity in vitro. This is likely due to the rapid clearance of the peptide from the circulation by well-

known mechanisms of uptake into kidney, liver, and other tissues.

The bound structure of the peptides may serve as a guide in designing individual small molecule mimetics, or small molecule libraries to reselect against the EPOR. Small molecule EPO mimetics may have desirable pharmacological properties such as oral availability or the ability to be delivered trans-dermally, neither of which has been achieved for EPO. The general utility of this approach to the discovery of agonists of other cytokine receptors remains to be demonstrated.

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17. Single letter amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu, M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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33. sEPOR was immobilized in polystyrene wells (Immulon 4, Dynatech) on mAb179 at a 1:1 dilution of receptor harvest in binding buffer (RPMI, 1 percent v/v BSA, 0.02 percent w/v sodium azide). About 1000 random peptide library equivalents were incubated with the immobilized receptor for 2 hours at 4°C. The unbound phage particles were then removed by six cycles of washing with PBS and then shaking with PBS for 30 minutes at 4°C. Receptor-phage complexes were cleaved from mAb179 with 10 μg of bovine thrombin (ICN) per well, in a volume of 100 μl of binding buffer supplemented with 2.5 mM $CaCl_2$ at room temperature for 10 minutes. Treatment with thrombin had no effect on phage viability or infectivity (*35*). Furthermore, thrombin inactivation (by chelation of Ca^{2+} ions) and heat denaturation of receptor (65°C for 10 minutes—to release phagemids) had no effect on subsequent amplification of recovered phage particles and were thus omitted.
34. The gene for pVIII was chemically synthesized with the use of four overlapping oligonucleotides. The gene was inserted into the Nhe I and Hind III sites of the phagemid vector pBAD18 (*64*), placing the expression under the control of the araB promoter (plasmid p8V2). A cloning site consisting of two non-complementary Bst XI sites was included at the 5′ end of the gene for pVIII and was used to clone collections of degenerate oligonucleotides by the half-site primer approach as described (*5*). Library ON1203 was constructed in this vector and contained 2.4×10^9 individual recombinants.
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37. The following conditions were used for affinity enrichment. Round 1 consisted of immediate acid elution after removal of unbound phage by washing (eluted phage were amplified for input into round 2). In rounds 2 and 3, bound phage were incubated with 100 nM EPO at 4°C for 15 minutes, washed, eluted and amplified. In round 4, the procedure was the same as in rounds 2 and 3, except that EPO incubation was at room temperature. In round 5, the EPO incubation was at room temperature for 30 minutes.
38. Individual clones were randomly picked and tested for sEPOR binding by phage ELISA. Positive clones were then tested for their ability to remain bound to the sEPOR in the presence of EPO. Lower affinity peptides should dissociate from the receptor more rapidly. In general, an input of 1×10^8 to 5×10^8 phage per well was used in this assay. After the phage were bound to the immobilized receptor, the wells were washed with PBS and then either PBS or 100 nM EPO in PBS was added to duplicate wells and incubated for 15 minutes at 4°C, and again washed. The percentage of total ELISA signal (no EPO) remaining in wells receiving EPO was used to rank clones in potential order of affinity. Clone C2/11 was reduced almost to background in this assay.
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62. All animal procedures were approved by the Institutional Animal Care and Use Committee of the R. W. Johnson Pharmaceutical Research Institute.
63. Mice were injected subcutaneously with test compound in a volume of 0.5 ml. Animals were injected 48 hours later with 0.4 μCi of ^{59}Fe. Individual body weights were determined 24 hours later and blood samples (200 μl) were taken, in duplicate, a further 24 hours later. The percentage of ^{59}Fe incorporated was determined (cpm $\times$ 5 $\times$ W $\times$ 0.08 $\times$ 100/T, where cpm is counts per minute of individual sample; 5 is the conversion factor to 1.0 ml; W is weight of mouse in grams; 0.08 is the mouse blood volume factor; and T is total injected counts of ^{59}Fe).
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